Biochimica et Biophysica Acta 1833 (2013) 2293–2301

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Detection and quantification of endoplasmic reticulum stress in living cells using the fluorescent compound, Thioflavin T

Daniel R. Beriault [a,b], Geoff H. Werstuck [a,b,c,*]

[a] Thrombosis and Atherosclerosis Research Institute, McMaster University, Hamilton, ON, Canada
[b] Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada
[c] Department of Medicine, McMaster University, Hamilton, ON, Canada

ARTICLE INFO

Article history:
Received 4 January 2013
Received in revised form 16 May 2013
Accepted 20 May 2013
Available online 5 June 2013

Keywords:
Thioflavin T
Endoplasmic reticulum stress
Unfolded protein response
Detection

ABSTRACT

The endoplasmic reticulum (ER) plays a central role in the co- and post-translational modification of many proteins. Disruption of these processes can lead to the accumulation of misfolded proteins in the endoplasmic reticulum — a condition known as endoplasmic reticulum stress. In recent years, the association of endoplasmic reticulum stress with a number of disease pathologies has increased interest in the study of this condition. Current methods to detect endoplasmic reticulum stress are indirect and retrospective. Here we describe a new method to detect and quantify endoplasmic reticulum stress in live cells using Thioflavin T (ThT), a small molecule that exhibits enhanced fluorescence when it binds to protein aggregates. We show that enhanced ThT-fluorescence correlates directly with established indicators of unfolded protein response activation. Furthermore, enhanced ThT-fluorescence can be detected in living cells within 20 min of application of an endoplasmic reticulum stress-inducing agent. ThT is capable of detecting endoplasmic reticulum stress induced by distinctly different conditions and compounds, in different cultured cell types as well as in mouse tissue samples. Pre-treatment with a potent endoplasmic reticulum stress-reducing agent, 4-phenylbutyric acid, mitigates the enhanced ThT signal. This new tool will be useful in future research investigating the role of protein misfolding in the development and/or progression of human diseases.

1. Introduction

The endoplasmic reticulum (ER) plays a central role in lipid biosynthesis and maintenance of calcium homeostasis. In addition, the ER is responsible for the efficient trafficking of approximately one third of all proteins produced in a typical eukaryotic cell. Central to this function is the co- and post-translational processing of proteins, including disulfide bond formation and N-linked glycosylation, which are essential for proper protein folding [1]. Under conditions where the influx of nascent proteins exceeds the processing capacity of the ER, unfolded or misfolded proteins can accumulate and disrupt ER homeostasis — a condition known as ER stress. Conditions of ER stress activate the unfolded protein response (UPR), which is a cellular self-defense mechanism that functions to alleviate ER stress by decreasing protein synthesis, enhancing protein folding capacity, and increasing the degradation of irreversibly misfolded proteins [2,3].

ER stress and/or activation of the UPR has been implicated in the development of several human pathologies, including neurodegenerative disorders (Alzheimer, Parkinson), diabetes mellitus, obesity, cancer and cardiovascular disease [4,5]. Therefore it is possible that factors that are indicative of ER stress and UPR activation could act as diagnostic markers of disease development or progression. Furthermore, if ER stress plays a causative role in disease pathogenesis, then this pathway may contain targets for therapeutic intervention.

ER stress can be measured indirectly by monitoring the activation/upregulation of various components of the endogenous UPR. Typically this involves using qRT-PCR or immunoblotting techniques to detect changes in UPR mRNA and protein levels, respectively [6]. The advantage of this strategy is that the methods are well established, the required molecular tools are readily available, and when performed and analyzed correctly, the results are accurate and consistent indicators of ER stress. However, these procedures are retrospective in nature because the ER stress markers are quantified hours after the cells are harvested. In addition, these methods indirectly assess ER stress, through the measurement of specific components of the UPR. Finally, multiple UPR gene products have to be monitored because other, ER stress-independent, pathways can affect expression of individual UPR genes (see review [6]).

Alternative systems have been developed to detect UPR activation using exogenous sensors that are inserted into the cells of interest.

* Corresponding author at: Department of Medicine, Thrombosis & Atherosclerosis Research Institute (TaARI), David Braley Cardiac, Vascular and Stroke Research Institute (DB-CVSRI), 237 Barton Street East, Hamilton, ON L8L 2X2, Canada. Tel.: +1 905 521 2100x40747; fax: +1 905 575 2646.
E-mail address: Geoff.Werstuck@taari.ca (G.H. Werstuck).

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.05.020

These include the secreted alkaline phosphatase (SEAP), XBP-1-venus fusion constructs, and reporter constructs containing XBP-1 or ATF6 binding sites [7–10]. The SEAP and XBP-1-venus systems have been shown to work in vivo as well as in cultured cells. The limitation of all of these detection systems is that they require the introduction of an exogenous transgene reporter.

The growing appreciation of the role of ER stress in the development and progression of several important diseases together with the limitations of the currently available systems of ER stress/UPR detection underlies the need for a more versatile technique to rapidly detect and quantify misfolded proteins. Thioflavin T (ThT) is a small molecule with fluorescence properties that has been shown to bind selectively to protein aggregates, particularly β-sheets [11–14]. The objective of this study is to characterize and evaluate the effectiveness of ThT, as a tool to detect protein aggregates as a measure of ER stress levels in living cells.

2. Methods

2.1. Cell culture

Mouse embryonic fibroblasts (MEFs) and human hepatocarcinoma (HepG2) cells were cultured in 1× DMEM (Invitrogen, Burlington, ON) containing 10% FBS (Hyclone, Rockford, IL). Human aortic endothelial cells (HAECs) were cultured in medium 200 (Cascade Biologics, Portland, OR) containing 10% Low Serum Growth Supplement (Cascade Biologics). All cells were maintained at 37 °C in 5% CO_2. Cells were split with 1× trypsin into 6-well culture dishes and allowed to adhere overnight before incubation with specific treatments for 0–18 h. Cells were washed with 1× PBS and treatments were filter sterilized and diluted in culture media prior to application. ThT (Sigma, St. Louis, MO) was dissolved in 0.5 mL 70% ethanol and diluted in 5.5 mL media to create a 5 mM stock solution. The stock solution was further diluted in media to a final concentration of 5 μM. Glucosamine hydrochloride (Sigma) and 4-phenylbutyric acid (4PBA) (Science Lab, Houston TX) were diluted in media and adjusted to pH 7.2. DTT (Sigma) and thapsigargin (Sigma) were diluted in media, and palmitate (Sigma) was prepared in BSA and media as previously described [15].

2.2. Immunoblot analysis

Monoclonal antibodies to Grp78/94 (anti-KDEL, Stressgen Biotechnologies, Victoria, BC), Gadd153/CHOP (Santa Cruz, Santa Cruz, CA), and anti-β-actin (Sigma-Aldrich) were used for protein detection/quantification by immunoblot analysis. Total protein lysates from cell culture were normalized by Bradford assay, solubilized in 4× SDS-PAGE sample buffer and separated on SDS-polyacrylamide gels under reducing conditions. Blots were incubated with the appropriate primary antibody for 18 h at 4 °C and subsequently with an anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO, Burlington, ON). Blots were developed using the Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore, Billerica, MA), and visualized with the ChemiDoc XRS System (BioRad). Band densities were quantified using Image Lab software.

2.3. Fluorescence detection and quantification

Fluorescent images of live cells were taken at a 500 ms exposure under a 10× objective (N.A. 0.25) with an Olympus CKX41 microscope (Ex. BP460–490 nm, barrier Em. 520 nm) and Infinity1-3C camera and saved using Infinity Analyze software (Lumenera Corp., Ottawa, ON). Fluorescent images were converted to 8-bit before being quantified by threshold analysis on ImageJ. Cells from each image were manually counted to normalize the fluorescent data per cell. Signal intensity, and assay sensitivity, can be increased by using objectives with a higher numerical aperture. Confocal images of 4% paraformaldehyde/PBS fixed cells were taken with a 63× glycerol immersion lens on a Multiphoton Leica TCS SP5 confocal microscope (Dr. Ray Truant's Lab, McMaster University). The excitation and emission settings were: DAPI (Ex. MP laser 800 nm, Em. 410–530 nm), ThT (Ex. 458 nm, Em. 480–520 nm), and Alexa568 (Ex. 561 nm, Em. 575–645 nm). Each fluorescent channel was imaged sequentially, as opposed to simultaneously, to avoid channel overlap. Cells were fixed for 20 min at room temperature, briefly permeabilized (5–30 s) with 0.05% TritonX/PBS, blocked for 20 min with 5% BSA/PBS and incubated with 1:700 mouse anti-KDEL antibody/PBS overnight at 4 °C. Cells were PBS washed (3×), then incubated with 1:700 Alexa568 anti-mouse secondary antibody/PBS for 1 h at room temperature (protected from light). Cells were PBS washed (3×), then incubated with RTU DAPI for 1 min, and washed with dH20 (3×). Cells were incubated with 5 μM ThT for 10 min (do not wash), and mounted to slides using 2 drops of Fluoromount and stored at 4 °C in the dark.

2.4. Mouse tissue

Fresh frozen ApoE$^{-/-}$ mouse liver was sectioned, collected onto cover slides and stored at −20 °C. Prior to ThT staining, sections were allowed to thaw for 5 min and then fixed in 37% formaldehyde

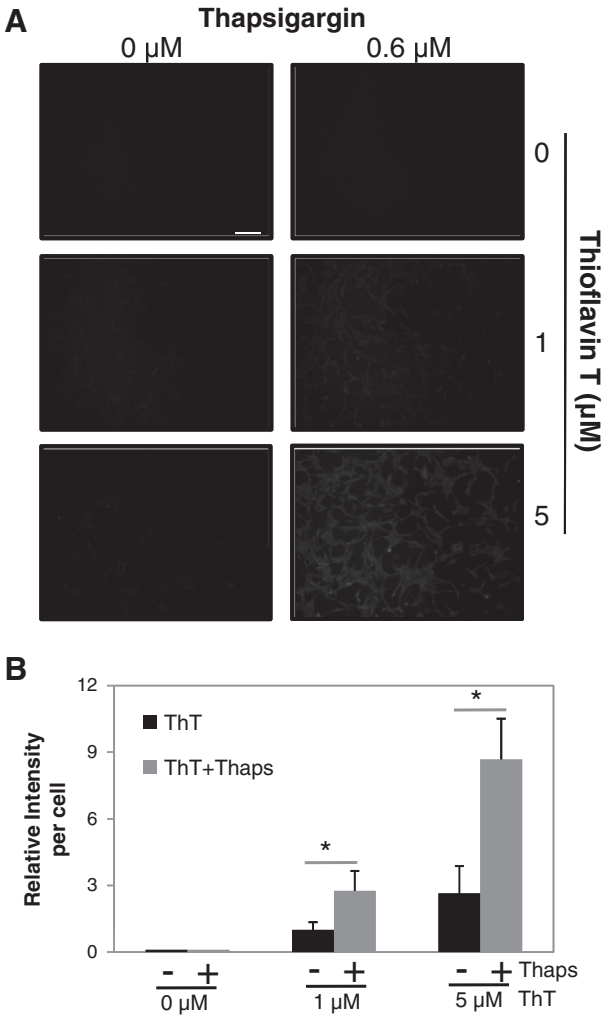

Fig. 1. Increased ThT fluorescence in the presence of thapsigargin. Mouse embryonic fibroblasts (MEFs) were treated with 0 or 1 μM thapsigargin (Thaps) and 0, 1 or 5 μM ThT for 18 h. A) Live cell images were captured and B) fluorescence intensity was determined and quantified. A significant increase in fluorescence was observed with 5 μM ThT. *$P < 0.05$, $n = 3$, Scale bar = 100 μm.

for 2 min at room temperature. Sections were then washed 2× with water, and incubated with fresh filtered 500 μM ThT in PBS for 3 min at room temperature. Slides were washed in water for 3 min. To preserve the fluorescence, slides were coated with 2 drops of Fluoromount (Sigma) and cover-slips were applied before imaging. The McMaster University Animal Research Ethics Board approved of all procedures.

2.5. Statistical analysis

Data are expressed as mean ± standard deviation. A significance of differences was determined using an unpaired t-test of equal variance. P values < 0.05 are considered statistically significant.

3. Results

3.1. The effect of ER stress on ThT fluorescence intensity

Our first aim was to determine the effect of ER stress on ThT fluorescence in living cells and also to identify the concentration of ThT that is required to give a significant and detectable signal. Mouse embryonic fibroblasts (MEFs) were cultured in the absence (0 μM) or the presence (1 μM) of the ER stress-inducing agent, thapsigargin [16], for 18 h. Increasing concentrations of ThT (0–50 μM) were added to the cultures. Low level background fluorescence (auto-fluorescence) was detected in the absence of ThT. In unstressed cells (0 μM thapsigargin), a linear increase in fluorescence was detectable with increasing ThT concentration (Fig. 1). Relative to controls, fluorescence was significantly enhanced in

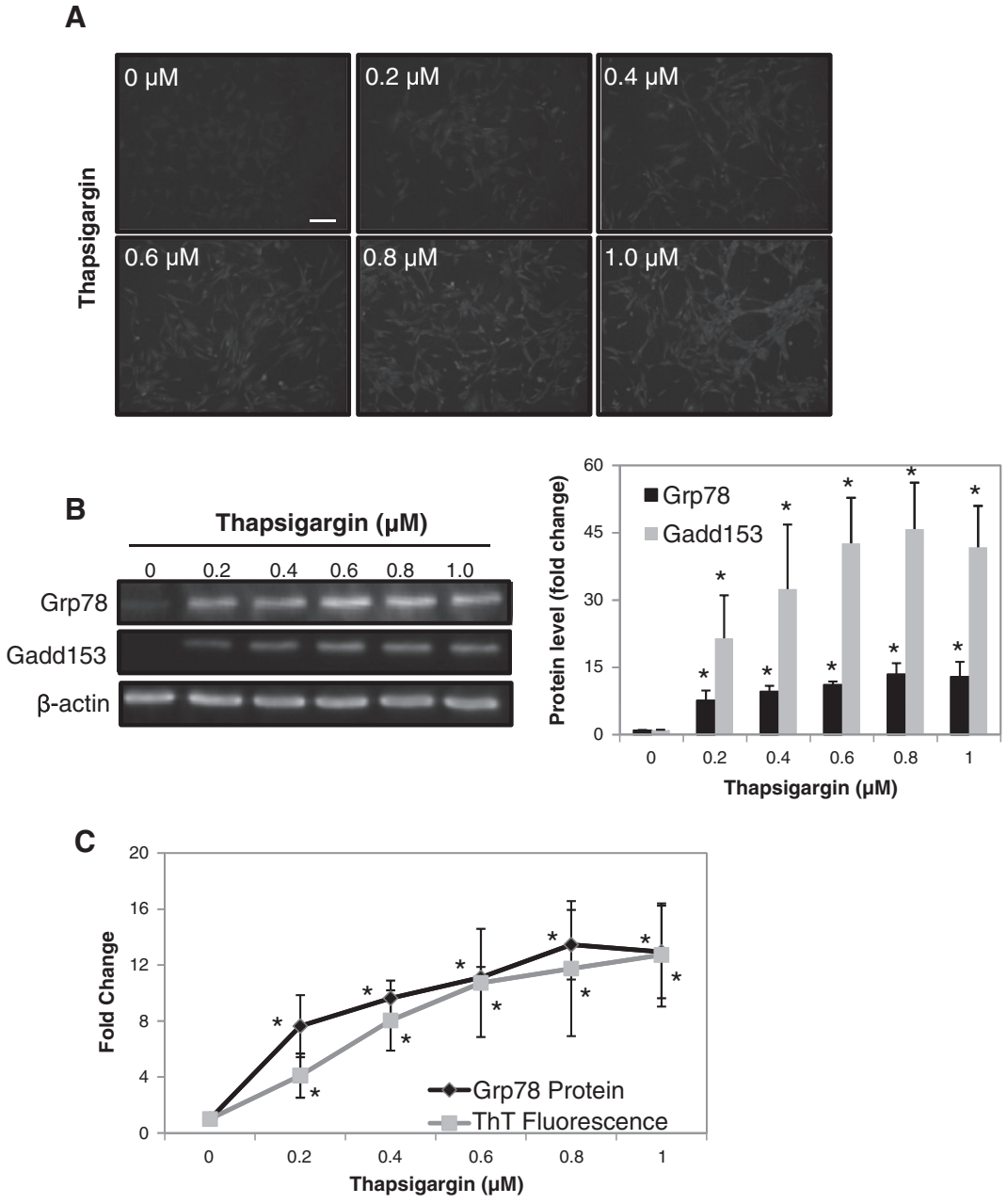

Fig. 2. ThT fluorescence intensity corresponds to ER stress-induced activation of the unfolded protein response. MEFs were treated with 0–1.0 μM thapsigargin and 5 μM ThT for 12 h. A) Live cell images were captured and fluorescence intensities were determined. B) Cells were harvested and total protein lysates were resolved for immunoblot analysis using antibodies specific for UPR proteins, Grp78 and Gadd153. C) The observed increase in Grp78 protein levels correlates directly with the measured increase in ThT fluorescence. *$P < 0.05$, $n = 3$, Scale bar = 100 μm.

thapsigargin treated cells. The maximum differential fluorescence (5.5 fold, $P < 0.01$) was observed in the presence of 5 μM ThT. Concentrations ≥50 μM ThT were toxic, and resulted in substantial cell death (data not shown). In all subsequent experiments, ThT was used at a concentration of 5 μM.

To determine if the intensity of fluorescence directly correlated to the level of UPR activation, MEFs were cultured in the presence of increasing concentrations of thapsigargin (0–1 μM) for up to 18 h. ThT (5 μM) was added to all cells. Images of living cells were captured, and fluorescence was quantified (Fig. 2A). The same cells were then harvested, proteins were resolved by SDS PAGE, and blots were probed with antibodies against established markers of ER stress, Grp78 and Gadd153 (Fig. 2B). As expected, increasing the concentration of thapsigargin resulted in increased Grp78 and Gadd153 protein levels. A similar, linear increase in ThT fluorescence intensity was also observed in these cells. This result suggests that there is a direct correlation between ThT fluorescence intensity and UPR activation in living cells (Fig. 2C).

3.2. The effects of chemical chaperones on ThT fluorescence

The observed direct correlation between ThT-dependent fluorescence and UPR activation is consistent with the possibility that ThT is interacting with accumulating misfolded proteins in the ER. To examine this possibility more closely, we treated cells with 0.6 μM thapsigargin in the presence or absence of 10 mM 4-phenylbutyric acid (4PBA), a chemical chaperone that has been shown to directly alleviate ER stress [17]. ThT and/or 4PBA treatment had no significant effect on fluorescence intensity (Fig. 3A,B) or the expression of UPR genes (Fig. 3C) in unstressed cells. Treatment with 4PBA significantly attenuated thapsigargin-induced Grp78 and Gadd153 expression levels, and was also associated with a significant decrease in ThT fluorescence. Similar results were observed when ER stress was attenuated using 400 μM tauroursodeoxycholic acid (TUDCA) (Supplementary Fig. 1). These data are consistent with ThT fluorescence acting as a direct indicator of misfolded proteins.

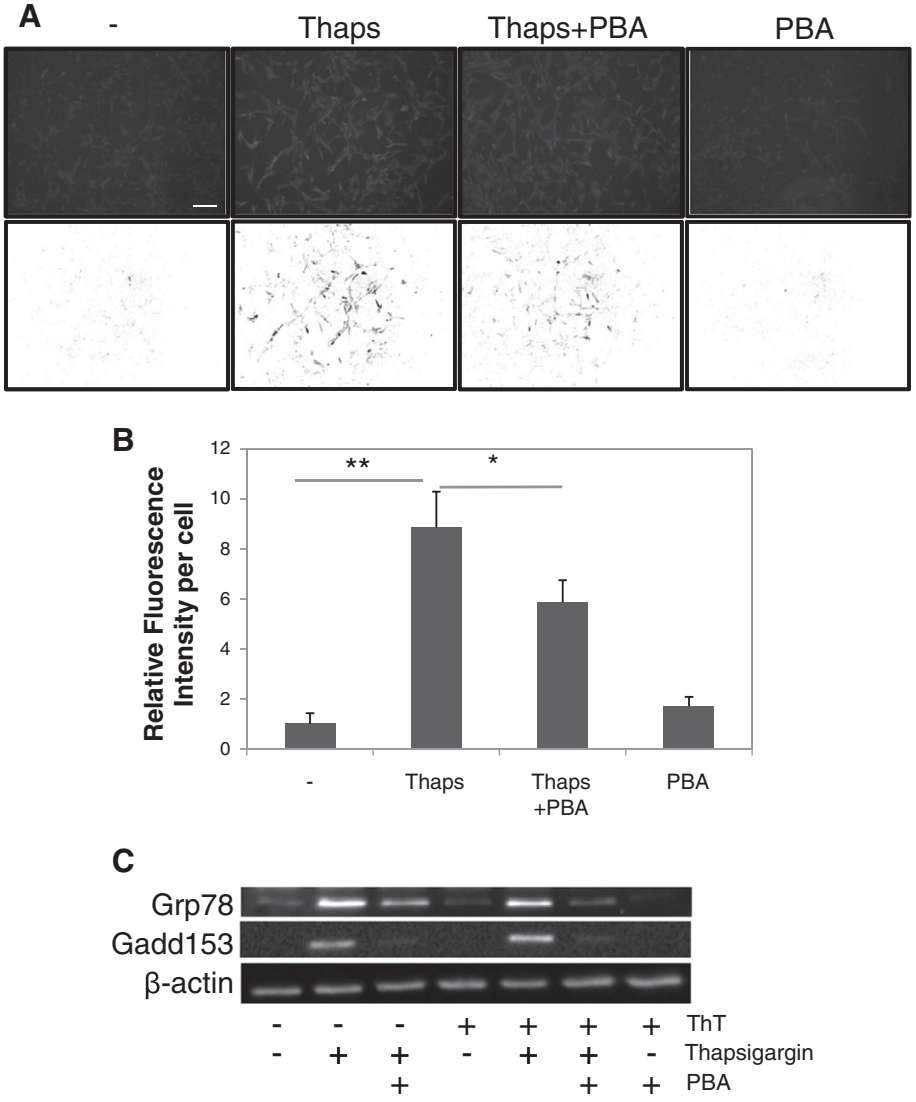

Fig. 3. Exposure to the chemical chaperone, 4PBA, reduces UPR activation and ThT fluorescence. MEFs were treated with 0 or 0.6 μM thapsigargin and 5 μM ThT as indicated for 12 h. A subset of cells was also treated with the chemical chaperone 4PBA (10 mM). A,B) Live cell fluorescent images were captured (top panel), processed by ImageJ thresholding to control for background fluorescence (bottom panel), and quantified. C) Total protein lysates were resolved by SDS-PAGE and analyzed by immunoblot using antibodies specific for Grp78, Gadd153 or β-actin, as a loading control. *$P < 0.05$, **$P < 0.01$, $n = 3$, Scale bar = 100 μm.

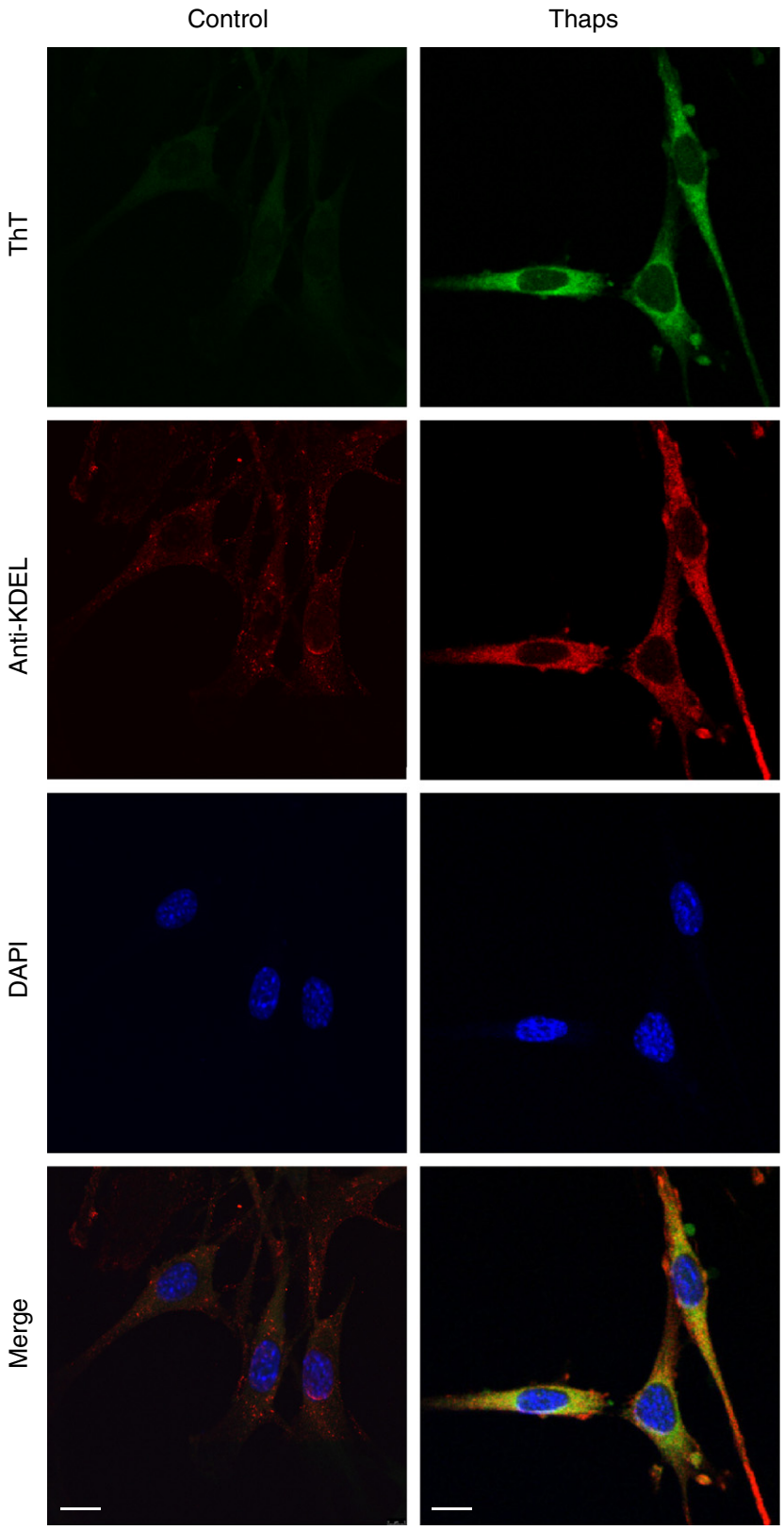

Fig. 4. Co-localization of ThT and KDEL in the ER of thapsigargin-treated MEFs. MEFs were treated with 0 or 1.0 μM thapsigargin for 8 h before visualization. Cells were fixed, probed with an anti-KDEL antibody, and stained with DAPI and 5 μM ThT, as indicated. Confocal images were captured using a Multiphoton Leica TCS SP5 confocal microscope. n = 4, Scale bar = 10 μm.

3.3. Enhanced ThT fluorescence co-localizes to the ER in stressed cells

In order to determine the subcellular localization of the ThT staining, MEFs were treated with 0 or 1.0 μM thapsigargin for 8 h and then stained, as described above, with ThT. The cells were subsequently probed with an anti-KDEL antibody that is specific for the ER resident chaperones Grp78 and Grp94. Multiphoton fluorescent confocal microscopy was used to visualize the staining (Fig. 4) and appropriate

controls (Supplementary Fig. 2). As expected, there is an increase in anti-KDEL staining in the perinuclear region of thapsigargin treated cells that is consistent with increased Grp78/94 protein concentration in the ER. Enhanced ThT-fluorescence in thapsigargin treated cells colocalizes to the ER. This result is consistent with ThT interacting directly with misfolded proteins in the ER.

3.4. The ability of different ER stress-inducing agents to enhance ThT fluorescence

Thapsigargin promotes ER stress by disrupting ER Ca^{2+} homeostasis. ER stress can also be induced by disrupting disulphide bond formation (DTT) [18], disrupting N-linked glycosylation (glucosamine) [19], or by altering the composition of the ER membrane (palmitate) [20]. To determine if enhanced ThT-dependent fluorescence was indicative of ER stress induced by distinctly different compounds, MEFs were treated with 2 mM DTT, 5 mM glucosamine, or 700 μM palmitate in the presence or absence of 10 mM 4PBA. Fluorescence was quantified and UPR protein markers analyzed. Results clearly show that each of the ER stress-inducing agents tested; i) promotes expression of Grp78 and Gadd153, and ii) enhances ThT fluorescence (Fig. 5A–C). Grp78/Gadd153 expression, and enhanced fluorescence, is attenuated, in each case, with the addition of the chemical chaperone 4PBA. This result suggests that ThT is a general indicator of ER stress.

3.5. The time course of enhanced ThT fluorescence

Indirect quantification of ER stress by immunoblot analysis of stress response proteins can only be performed after incubation times of 6 to 8 h because of the time required to induce changes in UPR protein levels through the activation of UPR gene expression [6]. To determine how soon, after application of an ER stress-inducing agent, that an increase in ThT fluorescence is detectable, MEFs were treated with ThT and 0.6 μM thapsigargin for 0–12 h. Images of live cells were captured and fluorescence intensity was measured. Results indicate that a significant increase in fluorescence, indicative of the accumulation of misfolded proteins, is detectable by 20 min after treatment with the ER stress-inducing agent (Fig. 6). This time corresponds to the phosphorylation of eIF2α, one of the earliest indicators of the activation of the UPR/PERK pathway [21,22].

3.6. The ability of ThT to detect ER stress in different cell types

The ability of ThT to detect ER stress in non-MEF cell types was investigated. A hepatocarcinoma cell line (HepG2) and primary human aortic endothelial cells (HAEC) were challenged with 1 μM thapsigargin, 2 mM DTT or 5 mM glucosamine for 12 h. ThT (5 μM) was added to each cell culture, images were captured and fluorescence intensities quantified (Fig. 7). A significant increase in fluorescence was observed in cells treated with the ER stress inducing agents. This suggests that ThT can detect ER stress in multiple cell types.

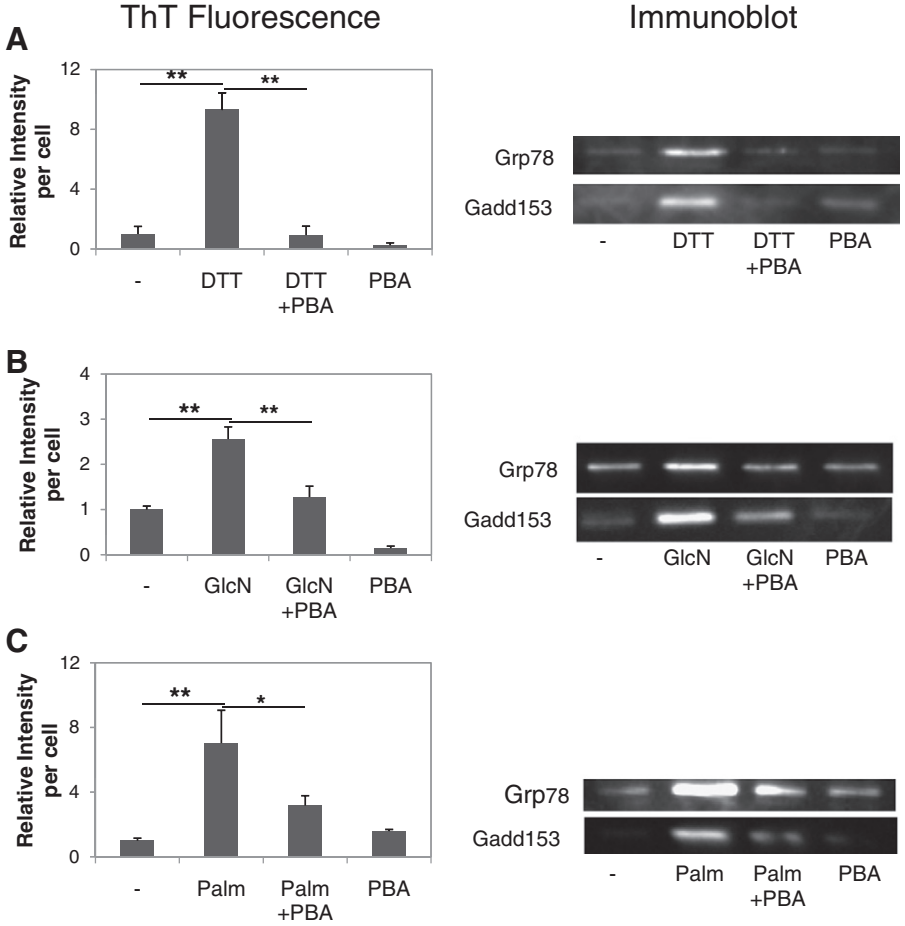

Fig. 5. Exposure to different ER stress-inducing agents enhances ThT fluorescence. MEFs were challenged with A) 2 mM DTT, B) 5 mM glucosamine (GlcN), or C) 700 μM palmitate (Palm), in the presence or absence of 10 mM PBA for 12 h, as indicated. ThT (5 μM) was added to all cells. Live cell fluorescent images were captured and quantified and total protein lysates were immunoblotted with antibodies against Grp78 or Gadd153, as indicated. *$P < 0.05$, **$P < 0.01$, $n = 3$.

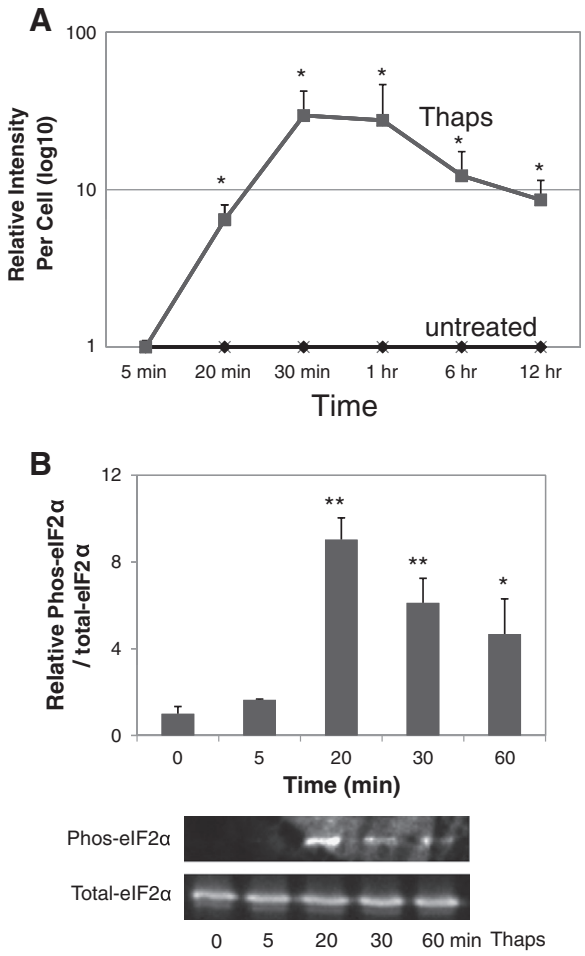

Fig. 6. Enhanced ThT fluorescence is detectable within 60 min. MEFs were challenged with 0.6 μM thapsigargin (Thaps) in the presence of 5 μM ThT. A) Live cell sequential images were captured and fluorescence intensity quantified 5, 20, 30, 60, 360 and 720 min after the addition of the ER stress inducing agent. B) MEF cells were harvested at the corresponding time points for immunoblot analysis of total and phospho-eIF2α. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$.

3.7. Detecting ER stress in mouse tissue

ThT has been used extensively to detect amyloid proteins in brain tissue [11,23]. To determine if ThT could be used to detect ER stress in tissues, we examined murine liver sections. We have previously shown that streptozotocin-induced hyperglycemia [24] or glucosamine-supplementation [25] can induce hepatic ER stress in ApoE$^{-/-}$ mice. In this experiment, frozen liver sections from glucosamine-supplemented mice were stained with ThT and compared to age and gender matched control mice. The results show significantly more ThT fluorescence in glucosamine-supplemented liver relative to the controls (Fig. 8). As observed in the cultured cell systems, inclusion of the chemical chaperone 4PBA [26] reduced the level of ThT fluorescence.

4. Discussion

Thioflavin T (4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N, N-dimethylaniline chloride) is a benzothiazole dye that exhibits enhanced fluorescence upon binding to proteins that are rich in β-sheet structures [11]. ThT has been shown to bind to amyloid fibrils containing "cross-β" structures consisting of laminated β-sheets whose strands run perpendicular to the fibril. Such structures are associated with Alzheimer's and prion diseases as well as systemic amyloidoses [23]. Upon binding to these structures, ThT exhibits a shift in emission maximum (from 445 nm to 482 nm) as well as an increase in fluorescence intensity of several orders of magnitude [12,13]. Together, these characteristics have made ThT a commonly used dye, both in vitro and in vivo, in the detection, diagnosis and analysis of amyloid fibrils and their associated pathologies in brain tissue and pancreatic islets [14,27,28].

Studies investigating the specificity of ThT have revealed that, in addition to amyloids, ThT can interact and bind to hydrophobic pockets in globular proteins. In particular, interactions with the α-helices of acetylcholinesterase [29], and the hydrophobic pocket of human serum albumin [30] are well documented. Furthermore, reports have shown that ThT can promote [31] or inhibit [32] protein aggregation suggesting that, under certain conditions, the interaction of ThT with protein structures can actively alter the folding process. Recently, ThT has been shown to interact with, and stabilize, DNA oligomers containing telemetric sequences in a pH and potassium-dependent manner [33]. The fluorogenic properties of ThT, together with its ability to bind to non-amyloid structures suggest that we are only beginning to understand the utility of this small molecule.

In a typical cell, one third of all of the proteins that are synthesized, are processed in the ER. ER stress is a condition in which the capacity of the ER resident folding machinery is overwhelmed by the load of nascent proteins. This results in the accumulation of misfolded proteins and the subsequent activation of the UPR [2,3]. The UPR is initiated by three different trans-ER membrane proteins, PKR-like ER Kinase (PERK), Inositol-Requiring Enzyme (IRE)-1 and Activating Transcription Factor (ATF)-6, which act as sensors of misfolded proteins. Upon activation these factors act in concert to; i) reduce the load of nascent proteins that require folding by inhibiting protein translation, ii) increase the protein processing capacity of the ER by specifically enhancing expression of chaperones and foldases, and iii) increasing the capacity of the cells to degrade terminally misfolded proteins [2,3].

Generally ER stress is detected indirectly by measuring the levels of specific UPR factors. Commonly measured indicators of UPR activation include; phosphorylated PERK, phosphorylated eIF2α, Gadd153/CHOP, ATF4, Grp78/BiP, Grp94, calreticulin, and protein disulphide isomerase (PDI). These markers are usually detected by immunoblot or immunohistochemistry [6,34]. ER stress-induced XBP-1 splicing and UPR gene expression can also be quantified using RT-PCR. Although these detection techniques have been successfully employed in many studies, they do have several limitations; i) mRNA isolation/RT-PCR and immunoblot analysis can be time consuming and analysis is retrospective, ii) the sample being analyzed is generally destroyed by the process of analysis, iii) other metabolic pathways can activate specific components of the UPR in the absence of ER stress thereby giving a false positive, iv) different cells/tissues express different sets of UPR genes and the detection methods have to be modified accordingly, and finally, v) all of these procedures are indirect measures of ER stress.

Our results suggest that ThT fluorescence is a viable new method to detect and quantify ER stress. The measured increase in ThT-fluorescence directly correlates to the degree of UPR activation, as quantified using more traditional assay systems. ThT has several apparent advantages over traditional methods including; i) enhanced ThT fluorescence is detectable 20 min after application of an ER stress-inducing agent, ii) ThT fluorescence can be continuously monitored and quantified in living cells over time, iii) ThT fluorescence is a more direct measure of protein aggregation, and iv) ThT detection is effective in different cell types, that are challenged with a range of different ER stress-inducing agents and conditions.

At the present time, we do not know if the enhanced ThT fluorescence that is observed under conditions of ER stress is attributable to specific misfolded proteins or protein structures, or if the fluorescence is a result of non-specific binding to protein aggregates. Although we do present data showing that enhanced ThT fluorescence co-localizes with ER resident KDEL containing proteins under conditions of ER stress, we do not directly show that ThT is interacting

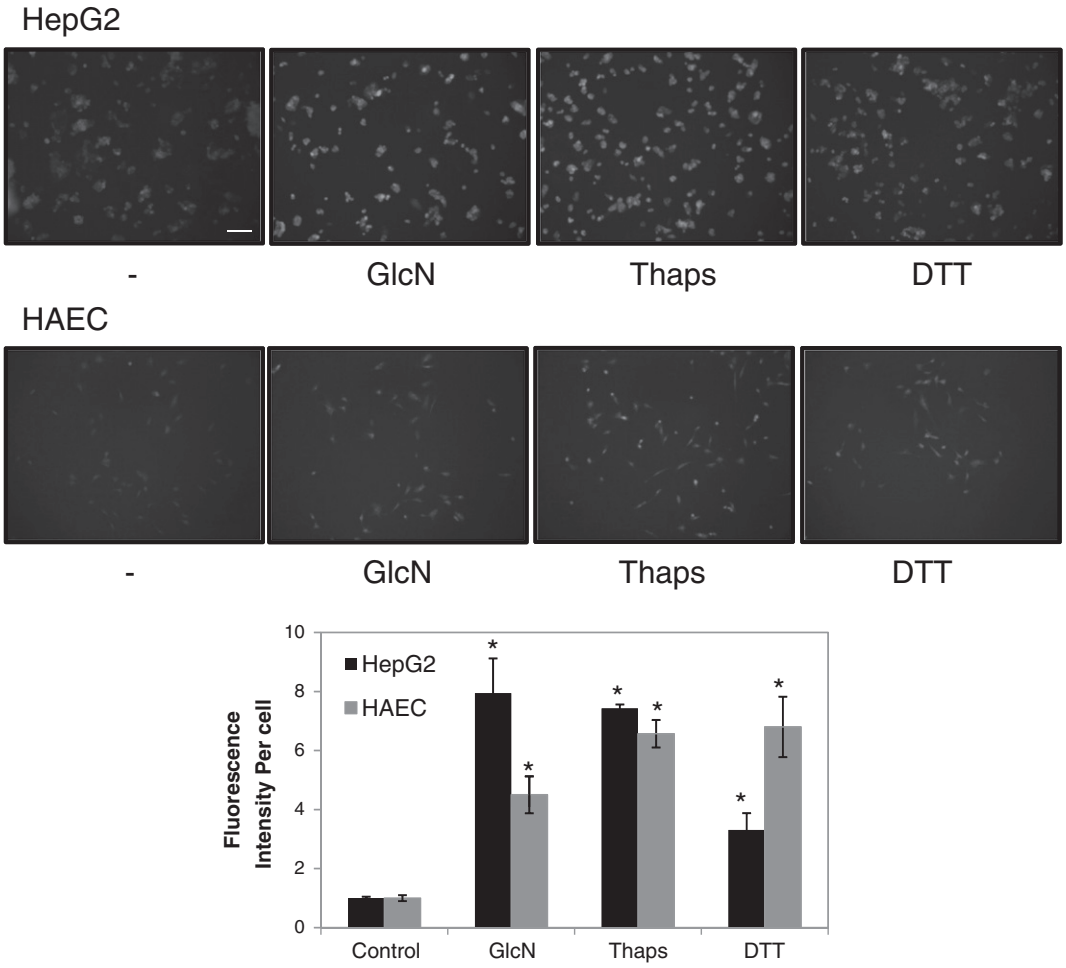

Fig. 7. ER stress promotes enhanced ThT fluorescence in different cell types. Human hepatocellular carcinoma cells (HepG2) and human aortic endothelial cells (HAEC) were exposed to 5 mM glucosamine (GlcN), 0.6 μM thapsigargin (Thaps) or 2 mM DTT as indicated and live cells were imaged after 6 h and quantified. *$P < 0.05$, $n = 3$, Scale bar = 100 μm.

with misfolded proteins in the ER. Previous reports have characterized the ability of ThT to bind to protein aggregates [11–14,32]. It should also be noted that ThT is not specifically localized to the ER and the accumulation of misfolded proteins in the cytosol, or in other subcellular organelles, may induce enhanced ThT fluorescence. Furthermore, conditions of unconventional UPR that selectively activate only one ER stress sensor (PERK, IRE, or ATF6) independently of protein misfolding, will likely not induce enhanced ThT fluorescence.

5. Conclusions

Our results indicate that ThT fluorescence can be used to directly detect, monitor and quantify ER stress levels in living cells and in animal tissues. This technique has several advantages over the current established methods used to measure ER stress levels; it is inexpensive, allows for rapid analysis in living cells and in real time. Evidence supporting a role for ER stress in the development of human conditions and diseases including Alzheimer's, Parkinson's, diabetes mellitus, obesity, cancer and cardiovascular disease has increased interest in the detection of misfolded proteins in the ER. One potential application of ThT may be the detection and quantification of ER stress in tissues of cancer patients. A recent clinical study has found that the expression of Grp78 and CHOP in visceral adipocytes predicts endometrial cancer progression and patient survival [35]. Future studies will target this and other potential applications of ThT as a novel ER stress-sensing tool.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.05.020.

Competing interests

The authors have no competing interests to declare.

Acknowledgements

We would like to thank Dylan DeMarco, Mark Bamberger, and Abby Emdin for technical support provided to this study. We would also like to thank Nick Caron and Dr. Ray Truant (McMaster University) for technical support on the Leica TCS Sp5 Confocal microscope. DRB is supported by funding from the Ontario Graduate Scholarship program. This study was funded by operating grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Ontario.

References

[1] A. Helenius, How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum, Mol. Biol. Cell 5 (1994) 253–265.
[2] H.L. Pahl, Signal transduction from the endoplasmic reticulum to the cell nucleus, Physiol. Rev. 79 (1999) 683–701.
[3] R.J. Kaufman, Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls, Genes Dev. 13 (1999) 1211–1233.
[4] S.J. Marciniak, D. Ron, Endoplasmic reticulum stress signaling in disease, Physiol. Rev. 86 (2006) 1133–1149.
[5] J.H. Lin, P. Walter, T.S.B. Yen, Endoplasmic reticulum stress in disease pathogenesis, Annu. Rev. Pathol. Mech. Dis. 3 (2008) 399–425.
[6] C.M. Oslowski, F. Urano, Measuring ER stress and the unfolded protein response using mammalian tissue culture system, Methods Enzymol. 490 (2011) 71–92.
[7] N. Hiramatsu, A. Kasai, K. Hayakawa, J. Yao, M. Kitamura, Real-time detection and continuous monitoring of ER stress in vitro and in vivo by ES-TRAP: evidence for systemic, transient ER stress during endotoxemia, Nucleic Acids Res. 34 (2006) e93.

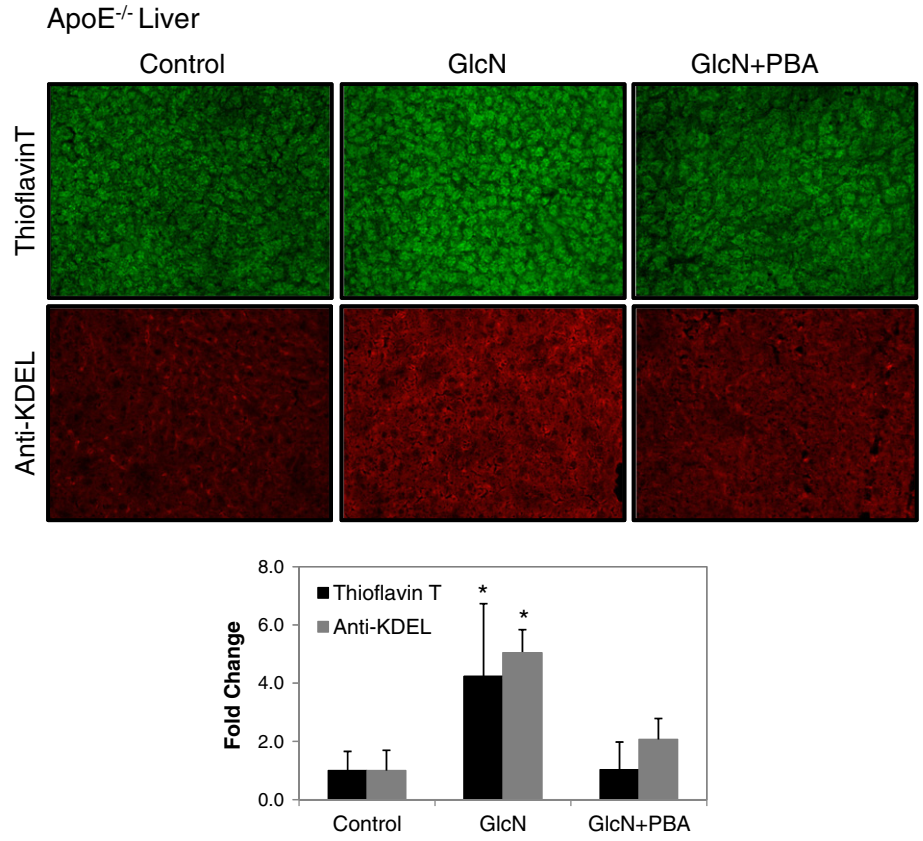

Fig. 8. Enhanced ThT fluorescence is detectable in mouse models of hepatic ER stress. Liver sections were prepared from 15 week old mice supplemented with glucosamine (5% w/v) [25] and/or 4PBA (1 g/kg body weight) [26] in the drinking water, starting at 5 weeks of age. Sequential sections were stained with 500 μM ThT or anti-KDEL as indicated and fluorescent images were captured. *$P < 0.05$, $n = 3$, Scale bar = 100 μm.

[8] T. Iwawaki, R. Akai, K. Kohno, M. Miura, A transgenic mouse model for monitoring endoplasmic reticulum stress, Nat. Med. 10 (2004) 98–102.
[9] A.H. Lee, N.N. Iwakoshi, L.H. Glimcher, XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response, Mol. Cell. Biol. 23 (2003) 7448–7453.
[10] Y. Wang, J. Shen, N. Arenzana, W. Tirasophon, R.J. Kaufman, R. Prywes, Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response, J. Biol. Chem. 275 (2000) 27013–27020.
[11] P.S. Vassar, C.F. Culling, Fluorescent stains, with special reference to amyloid and connective tissues, Arch. Pathol. 68 (1959) 487–498.
[12] H. Naiki, K. Higuchi, M. Hosokawa, T. Takeda, Fluorometric determination of amyloid fibrils in vitro using the fluorescent dye, thioflavin T1, Anal. Biochem. 177 (1989) 244–249.
[13] M. Lindgren, K. Sorgjerd, P. Hammarstrom, Detection and characterization of aggregates, prefibrillar amyloidogenic oligomers, and protofibrils using fluorescence spectroscopy, Biophys. J. 88 (2005) 4200–4212.
[14] E.M. Sigurdsson, Histological staining of amyloid-beta in mouse brains, Methods Mol. Biol. 299 (2005) 299–308.
[15] A.K. Busch, D. Cordery, G.S. Denyer, T.J. Biden, Expression profiling of palmitate- and oleate-regulated genes provides novel insights into the effects of chronic lipid exposure on pancreatic beta-cell function, Diabetes 51 (2002) 977–987.
[16] W.W. Li, S. Alexandre, X. Cao, A.S. Lee, Transactivation of the grp78 promoter by Ca2+ depletion. A comparative analysis with A23187 and the endoplasmic reticulum Ca(2+)-ATPase inhibitor thapsigargin, J. Biol. Chem. 268 (1993) 12003–12009.
[17] E. Erbay, V.R. Babaev, J.R. Mayers, L. Makowski, K.N. Charles, M.E. Snitow, S. Fazio, M.M. Wiest, S.M. Watkins, M.F. Linton, G.S. Hotamisligil, Reducing endoplasmic reticulum stress through a macrophage lipid chaperone alleviates atherosclerosis, Nat. Med. 15 (2009) 1383–1391.
[18] G. Negroiu, R.A. Dwek, S.M. Petrescu, Folding and maturation of tyrosinase-related protein-1 are regulated by the post-translational formation of disulfide bonds and by N-glycan processing, J. Biol. Chem. 275 (2000) 32200–32207.
[19] W. Qiu, R. Kohen-Avramoglu, S. Mhapsekar, J. Tsai, R.C. Austin, K. Adeli, Glucosamine-induced endoplasmic reticulum stress promotes ApoB100 degradation: evidence for Grp78-mediated targeting to proteasomal degradation, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 571–577.
[20] N.M. Borradaile, X. Han, J.D. Harp, S.E. Gale, D.S. Ory, J.E. Schaffer, Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death, J. Lipid Res. 47 (2006) 2726–2737.
[21] W. Yan, C.L. Frank, M.J. Korth, B.L. Sopher, I. Novoa, D. Ron, M.G. Katze, Control of PERK eIF2alpha kinase activity by the endoplasmic reticulum stress-induced molecular chaperone P58IPK, PNAS 99 (2002) 15920–15925.
[22] S. Dalal, C.R. Foster, B.C. Das, M. Singh, K. Singh, B-adrenergic receptor stimulation induces endoplasmic reticulum stress in adult cardiac myocytes: role in apoptosis, Mol. Cell. Biochem. 364 (2012) 59–70.
[23] Y. Xing, K. Higuchi, Amyloid fibril proteins, Mech. Ageing Dev. 123 (2002) 1625–1636.
[24] G.H. Werstuck, M.I. Khan, G. Femia, A.J. Kim, V. Tedesco, B. Trigatti, Y. Shi, Glucosamine-induced endoplasmic reticulum dysfunction is associated with accelerated atherosclerosis in a hyperglycemic mouse model, Diabetes 55 (2006) 93–101.
[25] D.R. Beriault, S. Sharma, Y. Shi, M.I. Khan, G.H. Werstuck, Glucosamine-supplementation promotes endoplasmic reticulum stress, hepatic steatosis and accelerated atherogenesis in apoE$^{-/-}$ mice, Atherosclerosis 219 (2011) 134–140.
[26] U. Ozcan, E. Yilmaz, L. Ozcan, M. Furuhashi, E. Vaillancourt, R. Smith, C. Gorgun, G. Hotamisligil, Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes, Science 313 (2006) 1137–1140.
[27] B.J. Cummings, C.J. Pike, R. Shankle, C.W. Cotman, Beta-amyloid deposition and other measures of neuropathology predict cognitive status in Alzheimer's disease, Neurobiol. Aging 17 (1996) 921–933.
[28] H.B. Rajamohamedsait, E.M. Sigurdsson, Histological staining of amyloid and pre-amyloid peptides and proteins in mouse tissue, Methods Mol. Biol. 849 (2012) 411–424.
[29] M. Harel, L.K. Sonoda, I. Silman, J.L. Sussman, T.L. Rosenberry, Crystal structure of thioflavin T bound to the peripheral site of *Torpedo californica* acetylcholinesterase reveals how thioflavin T acts as a sensitive fluorescent reporter of ligand binding to the acylation site, J. Am. Chem. Soc. 130 (2008) 7856–7861.
[30] P. Sen, S. Fatima, B. Ahmad, R.H. Khan, Interactions of thioflavin T with serum albumins: spectroscopic analyses, Spectrochim. Acta A Mol. Biomol. Spectrosc. 74 (2009) 94–99.
[31] M. D'Amico, M.G. Di Carlo, M. Groenning, V. Militello, V. Vetri, M. Leone, Thioflavin T promotes A(1–40) amyloid fibrils formation, J. Phys. Chem. Lett. 3 (2012) 1596–1601.
[32] S. Alavez, M.C. Vantipalli, D.J. Zucker, I.M. Klang, G.J. Lithgow, Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan, Nature 472 (2011) 226–229.
[33] J. Mohanty, N. Barooah, V. Dhamodharan, S. Harikrishna, P.I. Pradeepkumar, A.C. Bhasikuttan, Thioflavin T as an efficient inducer and selective fluorescent sensor for the human telomeric G-quadruplex DNA, J. Am. Chem. Soc. 135 (2013) 367–376.
[34] L. Qi, L. Yang, H. Chen, Detecting and quantitating physiological endoplasmic reticulum stress, Methods Enzymol. 490 (2011) 137–146.
[35] K. Matsuo, M.J. Gray, D.Y. Yang, S.A. Srivastava, P.B. Tripathi, L.A. Sonoda, E.J. Yoo, L. Dubeau, A.S. Lee, Y.G. Lin, The endoplasmic reticulum stress marker, glucose-regulated protein-78 (GRP78) in visceral adipocytes predicts endometrial cancer progression and patient survival, Gynecol. Oncol. 128 (2013) 552–559.